Isolation and Characterization of Bacteriophage SBX-1 and Its Bacterial Host, Both Endemic in Soybeans¹

J. M. DUNLEAVY AND N. V. R. URS

Agricultural Research Service, U.S. Department of Agriculture, and Department of Botany and Plant Pathology, Iowa State University, Ames, Iowa, 50010

Received for publication 29 January 1973

A phage, SBX-1, and its bacterial host, *Xanthomonas* sp. 1, were isolated consistently from roots, internal portions of stems, and leaves of soybean plants. Phage titer in leaves was highly variable. It was very low in seedlings, reached a maximum of 10^4 PFU/ml of sap after 11 weeks of plant growth and again dropped to very low levels. We isolated SBX-1 from plants of all 45 varieties studied, but not consistently from some. Plants of some varieties also carried *Xanthomonas* sp. 2, which was resistant to infection by SBX-1. The SBX-1 particle has a polyhedral head containing DNA of density 1.709. It has an edge-to-edge diameter of 80 nm and a tail length of 112 nm. The tail has a base plate and spikes. This is the first report of the extensive and continuous occurrence of a phage and its host bacterium in plants.

A phycovirus (AS-1) infectious to the blue-green algae *Anacystis nidulans* and *Synechococcus cedrorum* was recently described and characterized by Safferman et al. (15). The AS-1 particle contains double-stranded DNA in a polyhedral head 90 nm in diameter. A tail with base plate and spikes is attached to the head. The general morphology of AS-1 is similar to the SP50 phage of *Bacillus subtilis* (2) and the XP5 phage of *Xanthomonas pruni* (7), but no plant virus is known to be a phage.

There have been numerous reports of bacteria being associated with plants (5). Because we were able consistently to isolate certain bacteria from soybeans at specific stages of growth, we decided to search for a phage in soybeans by using bacteria isolated from soybean tissue as possible phage hosts.

MATERIALS AND METHODS

Isolation, purification, and host-range studies. The virus was isolated from soybean (*Glycine max* [L.] Merr.) leaves by an enrichment technique (1). Six grams of leaves were blended in 20 ml of potassium phosphate buffer (0.01 M, pH 7). The slurry was passed through gauze and centrifuged at $5,860 \times g$ for 15 min. A portion of this sap was used for the

determination of virus titer in plants. The remaining sap was passed through a Seitz filter (2- μ m pore size) and finally passed through a Millipore filter (0.45- μ m pore size). The filtrate was added to flasks containing concentrated Trypticase soy broth (TSB) to a final concentration of 3% TSB. Each flask was inoculated with 1 ml of a suspension of 10^6 cells of an 18-h culture of *Xanthomonas* sp. 1. The cultures were incubated overnight in a shaker-incubator at 30 C, after which bacteria were removed by centrifugation ($6,000 \times g$ for 10 min), and the supernatant fluid was filtered through a Millipore filter (0.45 μ m). The filtrate was used for phage assay.

The phage was obtained by transferring 1 ml of a suspension of 10^6 cells of host bacteria in TSB to side-arm flasks, each containing 200 ml of 0.25% TSB. The cultures were incubated at 37 C with gentle shaking, and the optical density (OD) was monitored at 30-min intervals at 540 nm. When OD reached 0.5 to 0.6, phage (MOI = 3) was added and enough calcium acetate was added to bring the final concentration to 10^{-3} M. The bacteria were considered lysed at OD 0.1 to 0.3. The remaining bacteria were removed by centrifuging at $4,080 \times g$ for 10 min. The supernatant fluid was passed through a 0.45- μ m Millipore filter and warmed to 37 C, and 1 mg each of DNase and RNase was added. The mixture was held at 37 C for 30 min and chilled to 10 C. The phage was pelleted by centrifuging at $106,000 \times g$ at 20 C for 1 h. The pellets were reconstituted in 3.5 ml of suspension medium (6 ml of 1 M Tris buffer at pH 7.5, 0.12 g of MgSO_4 , 4.0 g of NaCl, 0.05 g of gelatin, 1 liter of distilled water). This preparation was used for inoculum and other routine laboratory uses.

¹ Joint contribution from the Agricultural Research Service, U.S. Department of Agriculture, and as Journal Paper No. J-7246 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, 50010. Project 1179.

The phage was further purified for study by electron microscopy and for DNA isolation by density gradient centrifugation. The resuspended pellet was brought to initial buoyant density (1.5 g/cm^3) by adding CsCl and monitoring buoyant density by refractometer. This mixture was centrifuged in a swinging bucket rotor at $165,000 \times g$ at 20°C for 24 h. The gradients were fractionated, and fractions containing the phage were dialyzed against 1 liter of 1 M BPES buffer at pH 7.0 (1.0 M NaCl , 0.006 M Na_2HPO_4 , 0.002 M NaH_2PO_4 , 0.001 M EDTA). Phage susceptibility of bacterial hosts was tested on Trypticase soy agar medium with an overlay of the same medium containing 0.5% agar. Bacteria were tested as lawns on quadruplicate sets of phage dilution plates to 10^{-9} .

Internal tissue of stems and roots used for virus isolation was obtained by flooding the epidermis with 70% ethyl alcohol. Excess alcohol was blotted with sterile paper towels. Three-inch long segments of root or stem were split longitudinally and internal tissues were removed for assay.

Extraction of phage DNA. DNA was extracted by warming the purified phage suspension at 37°C and mixing with an equal volume of a pH 7.0 solution containing 10 M NaClO_4 and 10^{-3} M EDTA . This solution was dialyzed for 24 h at 50°C against 500 ml of 1 M BPES buffer at pH 9.0. The dialysis bag was soaked in a 64% solution of ZnCl_2 for 15 min and thoroughly rinsed before use. A 1-mg amount of DNase-free Pronase was added to the phage suspension, and dialysis was continued for 36 h against fresh buffer replaced at 12-h intervals. DNA thus prepared was stored at 4°C .

Extraction of bacterial DNA and determination of concentration of phage and bacterial DNA. DNA was isolated from the host bacterium by using the method outlined by Marmur (11). DNA concentration of the phage and host bacterium was determined by the method of Burton (3). Ultraviolet absorbancy of a 0.1 dilution of phage and bacterial DNA in standard saline-citrate solution (0.15 M NaCl , 0.015 M trisodium citrate) was scanned from 220 to 320 nm with a Beckman DBG spectrophotometer.

Buoyant density of phage particle, bacterial and phage DNA. The buoyant density of the phage and bacterial DNA and the intact phage particle was determined with a Beckman model E analytical centrifuge by using the procedure of Mandel et al. (8). The DNA of *Staphylococcus* phage 112, used as a reference, was kindly supplied by P. A. Pattee (Department of Bacteriology, Iowa State University). *Staphylococcus* phage 81 (12) was used as a reference for buoyant density determination of the intact phage particle.

One-step phage growth experiment. Phage growth characteristics were determined by a one-step growth experiment (17). Phage titer was adjusted to 10^8 PFU/ml, and the concentration of bacterial host cells was adjusted to 10^8 cells/ml at the beginning of the experiment. Adsorption was allowed to proceed for 15 min at 30°C , and samples were diluted 10^{-2} and 10^{-4} in TSB in test tubes at 30°C . Subsamples were drawn from these tubes at 2- to 3-min intervals for 1 h.

Adsorption experiment. The phage (10^8 PFU/ml) was adsorbed at 30°C on host cells (10^8 /ml) in TSB at a MOI of 1. The concentration of unadsorbed phage was determined at 2-min intervals for 12 min as described in the one-step phage growth experiment, except that samples were shaken with a few drops of chloroform and allowed to stand for 5 min before the final sample was drawn and plated.

Electron microscopy. The phage was suspended in pH 7.0 phosphate buffer, mounted on Formvar-coated, 100-mesh grids, and negatively stained with 4% uranyl acetate. Solutions were handled gently to prevent phage breakage. Two types of phage preparations were examined: (i) after initial concentration of the particles, and (ii) after further purification through density gradient centrifugation. Phage head dimensions were obtained by taking the mean of 10 measured heads. The electron microscope was calibrated by using a diffraction grating replica having 54,800 lines per inch.

RESULTS

Source and host range. The SBX-1 phage first was isolated from leaves of field-grown soybeans. We isolated 10 bacteria with distinct colony characteristics and assayed them for infectivity of phage in plant sap from leaves. One of the bacterial isolates was infected. Plaques, 1 to 3 mm in diameter, formed overnight on a lawn of the susceptible host, designated *Xanthomonas* sp. 1.

FIG. 1. One-step growth curve of SBX-1.

TABLE 1. Bacteria tested for susceptibility to SBX-1 phage

Susceptible	
<i>Xanthomonas</i> sp. 1 ^a	
Resistant	
<i>Bacillus subtilis</i> ^a	<i>Xanthomonas malvacearum</i>
<i>Escherichia coli</i>	<i>X. phaseoli</i>
<i>Pseudomonas fluorescens</i>	<i>X. phaseoli</i> var. <i>sojensis</i> ^a
<i>P. glycinea</i> ^a	<i>Xanthomonas</i> sp. 2 ^a
<i>Pseudomonas</i> sp. ^a	<i>X. pruni</i>
<i>Serratia marcescens</i> ^a	
<i>Staphylococcus aureus</i>	

^a Isolated from soybean plants.

FIG. 2. A, Tailless phage particles from a concentrated, partially purified preparation of SBX-1. B, SBX-1 particle from a centrifuged cesium chloride gradient. The particle at top is an empty head of a phage. C, An SBX-1 particle showing the outline of the polyhedral head. All phage particles were negatively stained with uranyl acetate. The bar represents 100 nm.

We tested bacteria of five genera for susceptibility to phage SBX-1 (Table 1). Only *Xanthomonas* sp. 1, of the 12 species tested, was infected by the phage. Seven of the species tested were isolated from soybeans.

Phage adsorption and one-step phage growth experiments. Adsorption of SBX-1 to *Xanthomonas* sp. 1 followed the kinetics of a first-order reaction. Ninety-six percent of the phage was adsorbed in 12 min at 30 C and at a MOI of 1.

The one-step growth curve of SBX-1 (Fig. 1) was typical of bacteriophages. SBX-1 had a latent period of 17 min, a rise period of 11 min, and a burst size of 100 PFU/cell. Four percent of the phage was unadsorbed by host cells 15 min after the phage was added to the suspension of *Xanthomonas* sp. 1 cells.

Description and properties of the phage. In repeated attempts after purification, we observed only the heads of the phage at an infective titer of 10^{11} with the aid of an electron

FIG. 3. Drawing of SBX-1 phage particle with particle dimensions taken from electron photomicrographs.

TABLE 2. Properties of SBX-1 and its *Xanthomonas* host compared to *Staphylococcus aureus* strain 112 and *S. aureus* phage 81

Organism	Density of phage particle	Density of phage DNA	Density of bacterial DNA	GC ^a of DNA (%)
Phage SBX-1	1.513	1.709		49.48
<i>Xanthomonas</i> sp. 1			1.714	55.37
Reference				
<i>S. aureus</i> phage 81	1.476			
<i>S. aureus</i> 112		1.693	1.693	

^a Guanine cytosine ratio.

microscope (Fig. 2A). The phage preparation was purified further by CsCl density gradient centrifugation. We obtained two bands in the gradient. The upper band contained no identifiable phage or phage parts, but the lower band contained intact phage particles (Fig. 2B and C).

SBX-1 has a polyhedral head and a relatively rigid tail (Fig. 2C and 3). The head is slightly longer than it is wide. The tail is 12.5 nm wide but is sharply constricted to 5 to 6 nm at the juncture of the head and tail, where the particles invariably broke in our early preparations. The tail has a base plate and tail spikes.

The SBX-1 particle was slightly more dense than the *Staphylococcus* phage 81 particle used as a reference (Table 2). The density of the phage DNA was 1.709 compared to 1.714 for the bacterium. These figures were the same on two separate determinations.

The phage was noninfectious to *Xanthomonas* sp. 1 after exposure to 70 C for 10 min or 65 C for 15 min. Only 3% of the phage PFU were infective after exposure to 60 C for 20 min.

Isolation from soybeans. We attempted to isolate SBX-1 from 45 soybean varieties during a 3-year period. Our first isolations were made from greenhouse-grown plants, and we were unable to isolate SBX-1 from plants of varieties resistant to bacterial pustule caused by *X. phaseoli* var. *sojensis* (Table 3). But we isolated the phage from some field-grown varieties resistant to bacterial pustule. We were unable to isolate the phage from greenhouse-grown Clark 63 (pustule resistant), but successfully isolated it from leaves, internal portions of stems, and roots of Clark (pustule susceptible) in 12 separate trials. We were unable to isolate the phage from Clark 63 plants grown in the field in 1969 and 1970. However, we did isolate it from Clark 63 in 1971, confirmed by an isolation the next week.

We invariably were able to isolate *Xanthomonas* sp. 1, the phage-susceptible host of SBX-1, from Clark soybeans. Under field condi-

TABLE 3. Some of the soybean varieties from which isolation of SBX-1 was attempted

Variety	Response to bacterial pustule ^a	Isolation of SBX-1		
		Field		Green-house
		1969	1970	
Adams	S	+		+
Amsoy	S	+		+
Chippewa	S	+	+	+
Chippewa rxp	R	-	+	-
Clark	S	+	+	+
Clark 63	R	-	- ^b	-
Corsoy	S	+		+
Ford	S	+	+	+
Hark	S	+		+
Harosoy	S	+	+	+
Harosoy rxp	R	-	+ - ^c	-
Hawkeye	S	+		+
Kent	S	+	+	+
Kent rxp	S	+	+	
Wayne	R	+	+	

^a Caused by *Xanthomonas phaseoli* var. *sojensis*; S = susceptible; R = resistant.

^b Isolated in 1971.

^c Isolated in July, but not in August.

tions, bacterial numbers ranged up to 10^3 /ml of sap. Likewise, we could invariably isolate *Xanthomonas* sp. 2, the phage-resistant bacterium, from Clark 63. Isolation of *Xanthomonas* sp. 1 and *Xanthomonas* sp. 2 from other varieties was variable.

We attempted to isolate SBX-1 from Adams, Illini, and T-135 soybean leaves at approximately weekly intervals from seedling emergence to near maturity. The phage was assayed by dropping sap directly on a lawn of *Xanthomonas* sp. 1 and by a series of dilution plates. In addition, we tested for presence of phage in sap by using the enrichment technique.

Phage titer was very low in all varieties from seedling emergence until 60 days after emergence, when it rose sharply in Adams and Illini and more slowly in T-135 (Fig. 4). Both Adams and Illini had a maximum titer of approximately 10^4 PFU/ml sap 76 days after emergence. Between 60 and 90 days after emergence, phage titer was sufficient in sap to cause lysis when added directly to a lawn of *Xanthomonas* sp. 1 on plates. Before and after this period, sap produced no lysis and the phage was not detected in dilution plates, but phage always was detected by using the enrichment technique.

DISCUSSION

This report is the first instance of a bacteriophage occurring extensively and continuously in plants. A similar virus particle, AS-1, infectious to blue-green algae was recently described by Safferman et al. (15). SM-1, a polyhedral virus

infectious to blue-green algae (14), is very similar to the tailless particles in our Fig. 2A, even to size (88 nm). LPP-1 also is a virus infectious to blue-green algae (13); this particle was first thought to be a simple polyhedron, but later was found to possess a short tail (16).

Several types of *X. pruni* bacteriophages have been differentiated on the basis of plaque morphology, host range, serology, lysogenicity, and other characteristics (4, 6, 9, 10). The detailed structure, chemical composition, and physicochemical properties have been described for only one *X. pruni* bacteriophage, XP5 (7). *X. pruni* is a plant pathogen, but none of these phages have been observed to occur naturally with the bacterium while it is infecting plants.

Numerous types of bacteria have been reported associated with plants aside from the symbiotic forms and those causing disease (5). No one has established that any bacterial forms are present consistently in the plant. Our research is unique in that we have established not only that the phage is always present in Clark, but that its host bacterium also is present and can be isolated readily. That phage numbers increase in a cyclic manner is of special significance and suggests that host numbers are increasing during the same period. Random sampling during this period showed *Xanthomonas* sp. 1 present in Clark leaves in unprecedented numbers for healthy plants.

Considering that Clark and Clark 63 are nearly genetic isolines (Clark 63 carries the genes for Phytophthora rot resistance, as well as for bacterial pustule resistance) and that both *X. phaseoli* var. *sojensis* and *Xanthomonas* sp. 2 are phage resistant, one is tempted to speculate that pustule resistance in the plant is related to the genetic difference between *Xanthomonas* sp. 1 and *Xanthomonas* sp. 2. Although we do not rule this out as a possibility, our present evidence clearly indicates that there is no relationship between presence or absence of either bacterium and plant resistance to bacterial pustule disease.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of P. A. Pattee with analytical centrifuge procedures and for providing phage and phage host DNA used for references. We also acknowledge the assistance of J. G. Holt and F. D. Williams with photomicrographs of the phage.

LITERATURE CITED

1. Baigent, N. L. 1967. Bacteriophage technique in phytopathology, p. 41-44. In R. N. Goodman (ed.), Proceedings of the first workshop on phytobacteriology. University of Missouri, Columbia.

FIG. 4. Cyclic increase and decrease of SBX-1 titer in leaves of three soybean varieties grown in the field.

2. Bradley, D. E. 1967. Ultrastructure of bacteriophages and bacteriocins. *Bacteriol. Rev.* **31**:230-314.
3. Burton, D. E. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315-323.
4. Crownover, R., J. D. Mandel, and A. Eisenstark. 1950. Studies of a bacterial virus active against *Xanthomonas pruni*. *Okla. Acad. Sci. Proc.* **31**:40-42.
5. Dunleavy, J. 1969. Consequences of intensive cultural practices on soybean diseases in the Corn Belt, p. 35-40. *In* J. A. Browning (ed.), *Disease consequences of intensive and extensive culture of field crops*. Iowa Agr. Home Econ. Exp. Sta. Spec. Rpt. 64.
6. Eisenstark, A., S. S. Goldberg, and L. B. Bernstein. 1955. Lysogenicity in *Xanthomonas pruni*. *J. Gen. Microbiol.* **12**:402-405.
7. Ghei, O. K., A. Eisenstark, C. M. To, and R. A. Consigli. 1968. Structure and composition of *Xanthomonas pruni* bacteriophage. *J. Gen. Virol.* **3**:133-136.
8. Mandel, M., C. L. Schildkraut, and J. Marmur. 1968. Use of CsCl density gradient analysis for determining guanine plus cytosine content of DNA, p. 184-195. *In* L. Grossman and K. Moldave (ed.), *Methods in enzymology*, vol. 12B. Academic Press Inc., New York.
9. Mandell, J. D., and A. Eisenstark. 1952. Bacterial viruses active against *Xanthomonas pruni*. Differentiation of genetic types. *Monthly Res. Rep. Office Naval Res.* April.
10. Mandell, J. D., and A. Eisenstark. 1953. Factors affecting plaque formation by *Xanthomonas pruni* bacteriophage. *Phytopathology* **43**:27-29.
11. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**:208-218.
12. Pattee, P. A., and J. N. Baldwin. 1961. Transduction of resistance to chlortetracycline and novobiocin in *Staphylococcus aureus*. *J. Bacteriol.* **82**:875-881.
13. Safferman, R. S., and R. E. Morris. 1963. Algal virus: isolation. *Science* **140**:679-680.
14. Safferman, R. S., I. R. Schneider, R. L. Steere, M. E. Morris, and T. O. Deiner. 1969. Phycovirus SM-1: a virus infecting unicellular blue-green algae. *Virology* **37**:386-395.
15. Safferman, T. O., T. O. Deiner, P. R. Desjardins, and M. E. Morris. 1972. Isolation and characterization of AS-1, a phycovirus infecting the blue-green algae, *Anacystis nidulans* and *Synechococcus cedrorum*. *Virology* **47**:105-113.
16. Schneider, I. R., T. O. Deiner, and R. S. Safferman. 1964. Blue-green algal virus LPP-1: purification and partial characterization. *Science* **144**:1127-1130.
17. Stent, G. S. 1963. *Molecular biology of bacterial viruses*. W. H. Freeman Co., San Francisco.